Techniques

Multiplex Real-Time Quantitative PCR to Detect and Quantify *Verticillium dahliae* Colonization in Potato Lines that Differ in Response to Verticillium Wilt

Z. K. Atallah, J. Bae, S. H. Jansky, D. I. Rouse, and W. R. Stevenson

First, fourth, and fifth authors: Department of Plant Pathology, University of Wisconsin, Madison 53706; second author: Department of Horticulture, University of Wisconsin, Madison 53706; and third author: United States Department of Agriculture–Agricultural Research Service, Vegetable Crops Research Unit, University of Wisconsin, Madison 53706.
Accepted for publication 5 February 2007.

ABSTRACT

Atallah, Z. K., Bae, J., Jansky, S. H., Rouse, D. I., and Stevenson, W. R. 2007. Multiplex real-time quantitative PCR to detect and quantify *Verticillium dahliae* colonization in potato lines that differ in response to Verticillium wilt. Phytopathology 97:865-872.

Potato early dying (PED), also known as Verticillium wilt, caused by *Verticillium dahliae*, is a seasonal yield-limiting disease of potato worldwide, and PED-resistant cultivars currently represent only a small percentage of potato production. In this study, we developed a real-time quantitative polymerase chain reaction (Q-PCR) approach to detect and quantify *V. dahliae*. The efficiency of the designed primer pair VertBt-F/VertBt-R, derived from the sequence of the β-tubulin gene, was greater than 95% in monoplex Q-PCR and duplex (using Plexor technology) procedures with primers PotAct-F/PotAct-R, obtained from the sequence of the actin gene, designed for potato. As few as 148 fg of *V. dahliae* DNA were detected and quantified, which is equivalent to five nuclei. Q-PCR detected *V. dahliae* in naturally infected air-dried potato stems and fresh stems of inoculated plants. Spearman correlations indicated a high correlation (upward of 80%) between *V. dahliae* quantifications using Q-PCR and the currently used plating assays. Moreover, Q-PCR substantially reduced the variability compared with that observed in the plating assay, and allowed for the detection of *V. dahliae* in 10% of stem samples found to be pathogen free on the culture medium. The described Q-PCR approach should provide breeders with a more sensitive and less variable alternative to time-consuming plating assays to distinguish response of breeding lines to colonization by *V. dahliae*.

Potato early dying (PED), also known as Verticillium wilt, is a persistent disease of potato (*Solanum tuberosum*) and is considered by many to be the major yield-limiting biotic factor in the irrigated regions of potato production in the United States (46,48,49). PED is caused most commonly by the ubiquitous soilborne fungus *Verticillium dahliae*, either alone or in a synergistic interaction with the nematode *Pratylenchus penetrans* (29,30,46, 48,49). The disease leads to a reduction in leaf area (2,51) and premature senescence of potato plants, which shortens the period of tuber bulking, subsequently reducing yields. Yield losses associated with PED may reach 50%, but are more commonly in the range of 10 to 15% (46,48,49).

Verticillium wilt is a monocyclic disease, and the pathogen survives between growing seasons as microsclerotia, which also serve as the principal means for pathogen dispersal on seed tubers, farm equipment, and in soil and water. Currently, PED is controlled mainly through fumigation in fields previously planted to potato, extended crop rotations, or by planting in fields never previously planted with potato (“virgin land”). A few potato cultivars (e.g., Ranger Russet and Bannock Russet) exhibit resistance to *V. dahliae* in the field, but currently represent a small percentage of the total U.S. area planted to potato, the majority of which is planted to susceptible cultivars (e.g., Russet Burbank and Russet Norkotah). Several wild and interspecific hybrids of *Solanum* spp. show significant levels of resistance to PED and currently are being investigated as sources of resistance (26–28,34).

Corresponding author: Z. K. Atallah; E-mail address: atila@plantpath.wisc.edu

doi:10.1094/PHYTO-97-7-0865

Commonly, the assessment of the response of potato breeding lines to PED is based on the colonization of plant tissues by *V. dahliae*. Indeed, numerous currently grown potato cultivars were selected based on the quantification of *V. dahliae* propagules in plant material (6,14,25–28,38,39,62), which relies on the use of semiselective culture media such as Tergitol-NPX (NPX) (6). The number of colonies of *V. dahliae* observed on the NPX medium 2 weeks after transfer is translated into the number of CFU, which is used as a quantitative measure of plant colonization (6,14,25–28,38,39,62). Although the detection of the pathogen in plant tissues is improved using polymerase chain reaction (PCR) methodologies compared with the culture medium assay (13,33,35–37), quantification is tedious and ambiguous (36). Alternatively, real-time quantitative-PCR (Q-PCR) allows for a simpler quantification, in addition to an increased sensitivity compared with conventional PCR assays (1,11,12,53,60,63,64).

Sequences of the internally transcribed spacer region (ITS) of the ribosomal DNA (rDNA) often are used to design PCR and Q-PCR primers to detect different fungal pathogens in potato tissues (11,12,32,47,56-58). Such targets with high copy numbers make the amplification and detection of fungal pathogens using PCR easy and reliable. However, the number of rDNA copies fluctuates with the age and stage of growth of the organism (44). Furthermore, nonorthologous copies of the ITS region within species and among strains within the same species are described in the literature (31). Such inconsistencies may affect the quantification of pathogen propagule numbers and potential comparisons among samples. Single-copy nuclear genes would provide specific primers, allowing for consistent pathogen quantifications regardless of age and growth stage.

The objective of this study was to develop a *V. dahliae* quantification methodology based on Q-PCR which can provide higher sensitivity, accuracy, and specificity compared with the NPX plating assay. The ability to detect and quantify pathogenic propagules soon after infection and prior to the appearance of PED symptoms would provide breeders with a tool to accelerate the selection process for resistance to PED and reduce the variability arising from plating assays.

MATERIALS AND METHODS

Isolate collection. DNA from 45 *V. dahliae* isolates, 9 other *Verticillium* spp., 17 *Fusarium* spp., and isolates of 10 different pathogens of potato or crops used in the rotation with potato was used to develop the intended Q-PCR primers. The *V. dahliae* isolates were chosen from various geographic locations (in the United States and other countries) and various hosts. A subset of isolates used to validate the specificity of the designed primer pairs is shown in Table 1. All *Verticillium* spp. DNA samples were obtained from the collection of D. I. Rouse and were used by Li et al. (33). All other DNAs were obtained by growing individual fungal and oomycete isolates in clarified V8 broth, prior to extracting DNA with FastDNA kit (MP Biomedicals, Irvine, CA), following the manufacturer's recommendations. DNA samples were quantified fluorometrically using Quant-IT PicoGreen double-stranded (ds)DNA (Invitrogen, Carlsbad, CA) and stored at 4°C until used.

Primer design. Sequences of functional protein-coding genes were generated for the purpose of designing primers specific for *V. dahliae* by sequence comparative analyses. Universal primer pairs were used to amplify fragments of six genes from DNA of 81 fungal and oomycetous isolates in the abovementioned collection. Those genes were elongation factor 1α (*ef1α*), calmodulin (*cal*), actin (*act*), *β-tubulin* 1 and 2, histone 3 (*H3*), and histone 4 (*H4*) (7,22). The sequences of these central function genes typically are highly conserved within species but polymorphic among species and genera. For the purposes of this study, only the sequences exhibiting polymorphisms that specifically differentiate *V. dahliae* from other species (most especially within the genus *Verticillium*) will be discussed hereafter.

PCR amplifications were conducted in a Bio-Rad iCycler thermocycler (Hercules, CA), one-half degree lower than the calculated melting temperature for the primers to maximize amplification specificity as predicted by Primer3 (50). The 20-μl amplification reactions included 1 ng of total purified culture DNA and 200 nM each primer, in Promega PCR Master Mix (Promega Corp., Madison, WI) (1.5 mM $MgCl_2$, 200 nM each dNTPS, and *Taq* Polymerase at 25 U/ml). Amplification products were visual-

TABLE 1. List of fungi, oomycetes, and bacteria used to design and evaluate primer pair VertBt-F/VertBt-R designed for the specific detection and quantification of *Verticillium dahliae*[a]

Pathogen (isolate)	Host	Isolate origin	Ct values
Verticillium dahliae (VD4)	Potato	Wisconsin	16.8
Verticillium dahliae (VD5)	Potato	Wisconsin	17.4
Verticillium dahliae (VD7)	Potato	Oregon	17.7
Verticillium dahliae (VD8)	Peppermint	Michigan	21.3
Verticillium dahliae (VD9)	Cotton	Syria	19.3
Verticillium dahliae (VD10)	Cotton	Australia	17.0
Verticillium dahliae (VD11)	Aslespas	Russia	20.6
Verticillium dahliae (VD12)	Cotton	Rhode Island	16.6
Verticillium dahliae (VD13)	Potato	Russia	17.0
Verticillium albo-atrum (VAa1)	Alfalfa	Wisconsin	NA
Verticillium albo-atrum (VAa2)	Alfalfa	Wisconsin	NA
Verticillium tricorpus (VT1)	…	…	NA
Verticillium tricorpus (VT2)	…	…	NA
Verticillium nigriscens (VN)	…	…	NA
Verticillium chlamidosporium (VCh)	…	…	NA
Verticillium cinnebarinum (VCn)	…	…	NA
Verticillium fungicola (Vf)	…	…	NA
Verticillium lateritium (Vla)	…	…	NA
Verticillium lecanii (Vle)	…	…	NA
Verticillium psaleotae (VP)	…	…	NA
Fusarium moniliforme (FM1)	Corn	California	NA
Fusarium subglutinans (FS)	…	…	NA
Fusarium proliferatum (FP)	…	…	NA
Fusarium oxysporum (FO1)	Barley	…	NA
Fusarium oxysporum (FO2)	Carrot	…	NA
Fusarium oxysporum (FO3)	…	…	NA
Fusarium oxysporum (FO4)	…	…	NA
Fusarium venenatum (NRRL 22196)	…	…	NA
Fusarium sambucinum (NRRL 22203)	…	…	NA
Fusarium graminearum (NRRL 34097)	…	…	NA
Fusarium oxysporum (NRRL 25891)	…	…	NA
Fusarium ventricosum (NRRL 22241)	…	…	NA
Fusarium eumartii (NRRL 22397)	…	…	NA
Fusarium coeruleum (NRRL 20434)	…	…	NA
Fusarium culmorum (NRRL 25475)	…	…	NA
Fusarium avenaceum (NRRL 25892)	…	…	NA
Fusarium acuminatum (NRRL 28654)	…	…	NA
Alternaria solani	Potato	Wisconsin	NA
Bipolaris maydis	Corn	Wisconsin	NA
Rhizoctonia solani	Potato	Wisconsin	NA
Colletotrichum coccodes	Potato	Wisconsin	NA
Phytophthora infestans (US-8 940480)	Potato	New York	NA
Phytophthora erythroseptica (Pery #347)	Potato	Wisconsin	NA
Pythium ultimum (Pult Med#2)	Potato	Wisconsin	NA

[a] Cycle threshold (Ct) values of the real-time quantitative polymerase chain reaction assay also are listed. NA = no amplification and … = information not available to authors.

ized in 0.8% agarose gels. Both DNA strands of products smaller than 700 bp were sequenced directly. PCR-amplified products were cleaned using AMPure magnetic beads (Agencourt, Beverly, MA) prior to labeling using ABI BigDye Terminator (version 3.1; Applied Biosystems; Foster City, CA), and excess labeling dyes was removed with CleanSEQ magnetic beads (Agencourt). Amplification products in excess of 700 bp were cloned prior to sequencing (in both directions) in the Topo TA Cloning kit (Invitrogen). Sequencing was performed at the University of Wisconsin Biotechnology Center, using the ABI 3730xl DNA Analyzer. Sequences were visualized in Chromas (Technelysium, Southport, Queensland, Australia), aligned using Clustal W (54,55), and the consensus sequence was edited in BioEdit (23).

Sequences from the target organisms, related species, genera, and other pathogens of potato and rotational crops were aligned in Clustal W. Primers were designed from areas polymorphic among species of the genus *Verticillium*, yet monomorphic within *V. dahliae*. Prospective primer sequences were evaluated using Primer3 to assess potential self-compatibility, melt temperatures, and gas chromatography clamping. Subsequently, candidate primers and amplicons were evaluated in Mfold (65) for potential secondary structures, which would reduce amplification efficiency. Moreover, care was taken to obtain primers for *V. dahliae* that could be duplexed with preexisting primers designed for the quantification potato DNA (1).

Evaluation of primer specificity. Primer specificity was tested using DNA from a subsample of the collection described above (Table 1). Q-PCR amplifications were conducted in triplicate on a Bio-Rad iCycler iQ real-time PCR system using the iQ Supermix SYBR-Green (Bio-Rad) with ≈1 ng of DNA and 200 nM each primer in 20 µl of total volume. The following amplification protocol was used: initial denaturation of 3 min at 95°C, followed by 40 cycles of 95°C for 10 s and 63°C (identified following temperature gradient amplifications) for 45 s. Melt curve analysis was used to distinguish potential primer dimers and nonspecific amplification products.

Primer efficiency. Amplification efficiency (efficiency = $10^{(-1/\text{slope})} - 1$) of each primer pair was tested on a sixfold serial dilution of *V. dahliae* DNA, starting at a concentration 14.8 ng/µl, with 1 µl of DNA added to a 20-µl Q-PCR reaction. Amplifications of *V. dahliae* target DNA were performed in the presence and in the absence of potato DNA. Potato DNA (25 ng) extracted from a disease-free and surface-disinfested tuber was added to the serial dilution of *V. dahliae* DNA. Q-PCR efficiencies were calculated using the Bio-Rad iCycler software (version 3.1). To determine the potential impact of the addition of potato DNA on the amplification of the target DNA, the slopes of the efficiency equations were compared using an analysis of covariance (ANCOVA) using PROC GLM in SAS (v. 9.0; SAS Institute, Cary, NC). Equations with similar slopes result in F statistics >1, indicating that variability is greater within an equation compared with between equations (8).

Field samples. Clones of various *Solanum* hybrids were planted at the University of Wisconsin Hancock Agricultural Experiment Station on Plainfield loamy sand soil (92% sand, 5% silt, 3% clay, and <1% organic matter) in a field routinely used to screen for resistance to *V. dahliae* in potato cultivars and breeding lines. This field has been planted continuously to potato for nearly 40 years and contains ≈50 CFU of *V. dahliae* per gram of soil. Additionally, ground rye seed infested with *V. dahliae* VCG4A isolate V18 were buried in the furrow with each tuber seed piece at planting (9 May 2005). Isolate V18 is a highly aggressive strain of *V. dahliae* that was isolated from severely affected potato plants in the same field and was used successfully in numerous studies of PED both in the field and in the greenhouse (2,3,26–28,33,43,52).

To generate the *V. dahliae* isolate V18 inoculum, rye kernels were soaked in distilled water overnight, drained, placed in a mushroom bag (1 kg/bag), and autoclaved twice (121°C for 70 min on two consecutive days). Subsequently, the bags were inoculated with 10 ml of a suspension of isolate V18 (6×10^6 conidia/ml), incubated for 2 months, and air dried. A Wiley mill with a 60-mesh screen was used to grind the colonized rye kernels to a powder with an infestation level of 200 CFU/g.

The planted potato plots were irrigated three times weekly. When plants appeared to be within 1 week of senescence, the basal portions of 112 stems measured from ground level to ≈10 cm up the stem were collected and air dried in order to measure colonization by *V. dahliae*. Air-dried basal stem segments were ground in a Wiley mill and separated into four 50-mg aliquots. Two 50-mg aliquots of each stem were plated on NPX medium (6) and colonies of *V. dahliae* were counted under a dissecting microscope after incubation for 2 weeks in the dark at room temperature. The remaining two ground stem 50-mg aliquots were used to extract DNA.

Greenhouse samples. In all, 12 plants of potato cv. Ranger Russet (PED resistant) and 12 plants of cv. Russet Norkotah (PED susceptible) were grown in the greenhouse. Nuclear seed of each cultivar was planted (10 February 2006) in a 1:1 vol/vol vermiculite and commercial potting mix. One week after emergence (7 March 2006), the plantlets were carefully removed from the pot and dipped for 10 min in a suspension (8×10^6 conidia/ml) of *V. dahliae* (isolate V18). Each plantlet then was transferred to a 15-cm pot filled with the soil mix described above. Growing conditions were 25 ± 2°C during the day and 18 ± 2°C at night, with a 16-h photoperiod. Plants were watered as needed and fertilized at the time of transplanting to the commercial potting mix using 20-20-20 commercial fertilizer (J.R. Peters, Allentown, PA). Two untreated control plants of each cultivar were dipped in water and otherwise treated similarly to the other plants in the trial. Furthermore, other plants of each of the two cultivars also were inoculated with *V. dahliae* isolate V18 and treated similarly, but were raised to maturity, along with noninoculated checks, to confirm expression of typical PED symptoms.

Four weeks after inoculation, a 5-cm-long portion at the base of the stem of each plant was excised, cut into small pieces, and divided into two groups. The crown in one group was squeezed to release the sap, which was used for the plating assay. For this assay, two 50-µl aliquots of the squeezed sap were spread onto NPX medium. Colony counting was completed after a 2-week incubation in the dark, at room temperature. A second group of crowns was ground in liquid nitrogen, using a mortar and pestle, and two 50-mg aliquots were used to extract DNA.

DNA extraction. All DNA samples were extracted using the FastDNA kit, using the manufacturer's recommendations. Subsequently, DNA extracts were cleaned up using the AMPure magnetic beads to eliminate remaining potential PCR inhibitors. All DNA samples were quantified fluorometrically using Quant-IT PicoGreen dsDNA and stored at 4°C.

Q-PCR amplifications. Q-PCR amplifications were conducted in triplicate (total of six amplifications performed on each stem), in a Bio-Rad iCycler iQ real-time PCR system using the iQ Supermix SYBR-Green (Bio-Rad) with ≈1 ng of DNA and 200 nM each primer in 20 µl of total volume. The following amplification protocol was used: initial denaturation of 3 min at 95°C, then 40 cycles of 95°C for 10 s and 63°C for 35 s. Melt curve analysis was used to distinguish potential primer dimers and nonspecific amplification products. The absence of PCR inhibitors in DNA samples was assessed by spiking each DNA sample with 10 pg of *Phytophthora infestans* DNA (isolate US-8 940480) and computing the cycle threshold (ΔCt) value ($\Delta Ct = Ct_{sample} - Ct_{control}$) (21). No natural infections with *P. infestans* have been observed in Wisconsin since the 2002 growing season. In all amplifications, DNA from pure cultures of each *V. dahliae* was used as the positive control and a no-template water sample as the negative control. The infection coefficient (IC), which is the ratio of $Ct_{host}/Ct_{pathogen}$

(59), was computed for each sample tested. The amounts of potato DNA were quantified using the primer pair PotAct-F/PotAct-R, designed to amplify the *act* gene in potato (GenBank accession no. X55751), as previously described (1).

A duplex Q-PCR assay of the selected two primer pairs was developed using Plexor Q-PCR System (Promega Corp.). Unlike the SYBR-Green assay, in which the incorporation of the fluorescent dye increases with the increasing number of copies of the DNA target, Plexor leads to a quenching of the dye and a reduction in fluorescence over time (20). One of the two primers is labeled with a fluorescent dye and modified with methylisocytosine (iso-dC) residue at the 5′ end, whereas the other primer is not. The dabcyl-iso-dGTP (iso-dG) present in the Q-PCR reaction mixture then is incorporated at the position complementary to the iso-dC label, effectively quenching the fluorescence over time. Primers VertBt-R and PotAct-R were labeled with fluorescein phosphoramidite (FAM; peak emission at 490 nm and peak excitation at 530 nm) and Redmond Red phosphoramidite (peak emission at 579 nm and peak excitation at 595 nm), respectively. Additionally, both primers were modified at the 5′ end with iso-dC. Primers VertBt-F and PotAct-F were not labeled or modified. Amplifications were performed in 25-μl reactions with 200 nM each primer, and using Plexor master mix (Promega Corp.). The following amplification protocol was used: initial denaturation of 2 min at 95°C, followed by 40 cycles of 95°C for 5 s and 61°C for 35 s. Unlike the TaqMan technology (Applied Biosystems), Plexor is amenable to melt curve analysis to distinguish potential nonspecific amplification products, including primer dimers.

Data analyses. To compare the results of the Q-PCR assay with colony counts, Spearman correlations were calculated using PROC CORR in SAS on both air-dried and fresh samples. A rejection of the null hypothesis (P value < 0.05) would indicate a lack of significant difference between IC versus CFU of *V. dahliae*.

RESULTS

Primer design, specificity, sensitivity, and efficiency. All sequences from strains of one species were monomorphic regardless of the host they were isolated from or geographic origin. Sequences of *act*, *ef1-α*, *H3*, *H4*, *β-tubulin* 1, and *cal* did not provide the needed polymorphisms to differentiate *V. dahliae* from the other species, especially *V. tricorpus* and *V. albo-atrum*. Conversely, sequences of *β-tubulin* 2 provided polymorphisms that separated *V. dahliae* closely from related species (e.g., *V. albo-atrum* and *V. tricorpus*), and these sequences were used to generate primers specific to *V. dahliae*.

The primer pair VertBt-F/VertBt-R amplified a 115-bp region and was designed from the sequence of *β-tubulin* 2 (Table 2), which provided high specificity to isolates of *V. dahliae* obtained from various locations and crops (Table 1). This primer pair did not amplify any of the 9 other *Verticillium* spp., 15 *Fusarium* spp. or pathogens of potato or rotational crops tested in this study (Table 1). Melt curve analysis indicated the presence of a single amplification product. This amplified product was cloned into a TOPO TA vector, and sequence analysis exhibited identity with the expected 115-bp target region in the *β-tubulin* gene (data not shown).

Primer efficiency exceeded 96% over six orders of magnitude of *V. dahliae* DNA concentration, when only DNA from pure culture was used (Table 3; Fig. 1). Furthermore, the efficiency exceeded 95% in the presence of 25 ng of potato DNA (Table 3; Fig. 1). The comparison of the slopes of both equations using ANCOVA indicated no significant difference ($P = 0.9255$) in the efficiency of the designed primer regardless of the presence of host DNA. The lowest concentration of *V. dahliae* DNA reliably amplified was 148 fg (1.48×10^{-6} ng) (Table 3). Lower concentrations of target DNA could not be quantified consistently.

Q-PCR amplifications of fresh greenhouse samples and air-dried field samples. ΔCt values ranged from –0.2 to +0.2 when fresh potato tissues were used, and ranged from –0.6 to +0.7 when air-dried tissues were used to extract DNA. The ranges are within the variability of iCycler IQ real-time PCR system. Hence, corrections of the Ct values were not needed.

Of the 12 inoculated Russet Norkotah plants, 10 were found to harbor *V. dahliae* when tested with Q-PCR and NPX plating assays (Table 4). Two plants escaped infection with *V. dahliae* and tested negative for *V. dahliae* using both assays. None of the Ranger Russet plants harbored the pathogen (Table 4). The Spearman correlation coefficient used to compare the CFU counts and IC was highly significant ($R^2 = 0.824$ and $P = 0.0005$). All the other plants that were left to go to maturity exhibited typical PED symptoms and died ≈2 to 3 weeks earlier than noninoculated plants.

All 112 air-dried interspecific potato hybrid samples evaluated in the field were infected with *V. dahliae* when tested with Q-PCR (Fig. 2). In comparison, 11 of the 112 samples tested negative with NPX plating assay (≈10%) (Fig. 2). The Spearman correlation coefficient on all 112 samples was highly significant ($R^2 = 0.703$ and $P < 0.0001$). When the 11 samples that tested positive with the Q-PCR and negative with the plating assay were excluded from the Spearman correlation analysis, the coefficient remained highly significant ($R^2 = 0.653$ and $P < 0.0001$).

Primer duplexing. *V. dahliae* and *S. tuberosum* DNA were amplified simultaneously using the two labeled and modified primer pairs using the Plexor Q-PCR System. Primer efficiencies over 5 orders of magnitude of serial dilution of DNA concentration remained elevated and were not affected by the duplexing and were 97% efficient for VertBt-F/VertBt-R and 98% efficient for PotAct-F/PotAct-R (Fig. 3).

DISCUSSION

Since the early 1980s, most studies aimed at quantifying the colonization of potato tissues with *V. dahliae* have relied on CFU-counting assays on culture media. Such plating methods were used to differentiate among cultivars and breeding lines in the

TABLE 3. Cycle threshold (Ct) values from six-point serial dilutions of *Verticillium dahliae* DNA from pure cultures in the presence or absence of potato (*Solanum tuberosum*) DNA

	Ct values for potato DNA added (25ng)	
V. dahliae DNA amount	No	Yes
14.8 ng	18.6	18.4
1.48 ng	21.1	20.3
148 pg	24.6	24.5
14.8 pg	28.3	28.0
1.48 pg	31.7	31.5
148 fg	35.4	35.3
Efficiency[a] (%)	96.1	95.2

[a] Real-time quantitative polymerase chain reaction efficiency values.

TABLE 2. Primers developed to detect and quantify *Verticillium dahliae* and *Solanum tuberosum* (potato) DNA

Target organism	Gene target	Primer	Sequence
Verticillium dahliae	*β-tubulin*	VertBt-F	AAC AAC AGT CCG ATG GAT AAT TC
	…	VertBt-R	GTA CCG GGC TCG AGA TCG
Solanum tuberosum	*act*	PotAct-F	TGA ACA CGG AAT TGT CAG CA
	…	PotAct-R	GGG GTT AAG AGG GGC TTC AG

selection for and study of resistance to PED (10,14,17,25–28, 45,62). Those assays were also employed to describe the epidemiology of the disease and colonization by the pathogen under various agricultural and environmental conditions (2–4,15,16,18, 19,24,40,43,48,52,61). Expression of PED symptoms is greatly affected by environmental and agricultural conditions (e.g., weather during the growing season, irrigation frequency, and nitrogen fertilization), and selection of breeding lines based solely on symptoms is generally discouraged. In this study, we developed an effective Q-PCR alternative to the tedious and time-consuming plating assays. NPX plates are incubated for at least 2 weeks and the medium allows for the growth of other species in the genus *Verticillium* (e.g., *V*. *albo-atrum* and *V*. *tricorpus*), which complicates pathogen quantification. Another complicating factor lies in the stochastic nature of the quantification using CFU counts, where a high variability within replicates of one sample commonly is observed (41). Furthermore, competition among colonies may affect *V. dahliae* quantification on culture media.

The sensitivity of detection of *V. dahliae* is greatly improved by the use of a PCR assay, as demonstrated by Li et al. (33), to detect as few as 50 target copies, and by Mahuku and Platt (35), who detected a larger number of microsclerotia. In this study, we were able to detect 148 fg of *V. dahliae* nuclear DNA using Q-PCR (Table 3), which is equivalent to approximately five haploid genomes of *Aspergillus nidulans* (5) or *V. dahliae* from noncruciferous hosts (9). With the knowledge that the *β-tubulin* gene is present in a single copy in the nuclear genome of fungi (42), these results indicate that the sensitivity of our Q-PCR assays allows for the detection of as few as five nuclei of *V. dahliae*, which is at least one order of magnitude lower than other PCR-based assays described in the literature (33,35). Other PCR-based approaches to quantify *V. dahliae* DNA relied on a visual end-point comparison of PCR-product band intensity on agarose gel to quantify *V. dahliae* by comparison with an internal control plasmid (13). The methodology described in the current study relies on a direct and real-time quantification of the DNA of *V. dahliae*, with no need to estimate DNA amounts against those of internal controls, and does not call for the use of gel electrophoresis. Also, the current approach provides for a means to compare amounts of host and pathogen DNA simultaneously, which other studies have not developed.

The primer pair VertBt-F/Vert-R exhibited high specificity to *V. dahliae* regardless of geographic origin or host plant infected (Table 1). Moreover, DNA from related species such as *V. albo-atrum* and *V. tricorpus*, and from other fungal and oomycetous pathogens, was not amplifiable with VertBt-F/Vert-R. We tried mixing known amounts of *V. dahliae* DNA with DNA from *Phytophthora infestans*, *P. erythroseptica*, *Pythium ultimum*, *Erwinia carotovora* subsp. *carotovora* and subsp. *atroseptica* (syn. *Pectobacterium carotovorum* and *P. atrosepticum*, respectively), and *Fusarium sambucinum*, but failed to measure significant deviation in Ct values compared with *V. dahliae* alone (data not shown). Additionally, the primer pair was used successfully to detect *V. dahliae* from several Verticillium-wilt-symptomatic samples of ash (*Fraxinus* sp.), maple (*Acer* sp.), and other ornamental species (data not shown). Although this study is aimed specifically at the detection and quantification of *V. dahliae* in potato tissues, the described primer pair VertBt-F/VertBt-R potentially could be used for similar quantifications in other hosts. This is especially true when considering that the origin of many of the *V. dahliae* isolates used to generate these primers were nonsolanaceous hosts. The primer pair also was successful in detecting *V. dahliae* in potato tubers showing typical *V. dahliae*-associated symptoms on the stem end section, as well as others not showing symptoms but collected from plants that exhibited PED symptoms (data not shown).

Results of the Q-PCR assay were highly correlated with CFU counts obtained from air-dried (R^2 = 0.82 and P = 0.0005) and fresh potato stem samples (R^2 = 0.70% and P < 0.0001). The results indicate that both methods are comparable in their ability to quantify *V. dahliae*. Nevertheless, Q-PCR amplifications of the two samples of infected potato tissues showed higher consistency of quantification compared with CFU counts. Similar to other related studies, the variability between the two samples tested by CFU counts was high (35,41), with the standard deviation on the CFU counts reaching 346 with a mean of 212 (data not shown). Conversely, Ct values from both DNA samples using Q-PCR fluctuated by ±0.5, which is within the normal variation of the iCycler iQ system, and a similar range of variation observed among the three amplification replicates from each DNA sample. Precision in quantification of the pathogen is critical for the selection of potato breeding lines and interspecific hybrids by breeders.

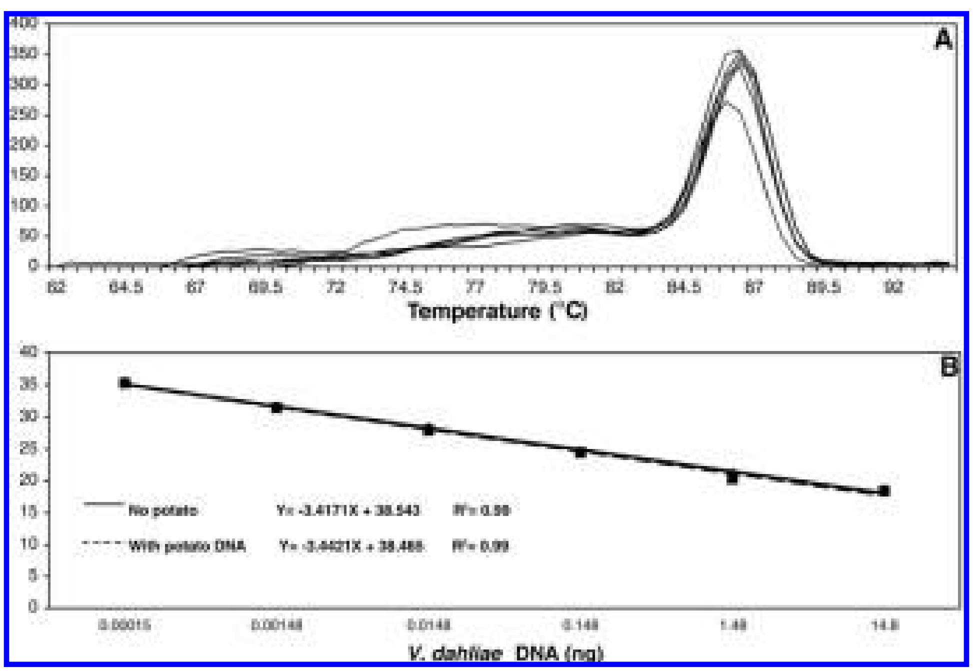

Fig. 1. A, Melt curve and **B,** efficiency curves of a six-point 10-fold serial dilution of *Verticillium dahliae* DNA in the presence or absence of potato DNA. Slopes of the efficiency curves are compared using analysis of covariance. RFU = relative fluorescence unit.

Additionally, for ≈10% of the air-dried potato stems tested, NPX plating failed to detect the presence of *V. dahliae*, unlike Q-PCR (Fig. 2). In addition to the high variance in CFU counts, such a high rate of type II error (i.e., the inclusion of false negatives) may have a substantial impact on efforts to select potato breeding lines based on their colonization by *V. dahliae*.

The primer pairs VertBt-F/VertBt-R repeatedly exhibited efficiencies >95% regardless of the presence or absence of large amounts of host DNA (Table 3; Fig. 1). The ANCOVA analysis on the slope of *V. dahliae* DNA alone (slope = –3.417) and of *V. dahliae* in the presence of 25 ng of potato DNA (slope = –3.442) yielded an F statistic of 7.01 (P = 0.9255) (Fig. 1). This indicates that the two slopes are not significantly different. Elevated efficiencies were measured over 6 orders of magnitude of DNA concentration exhibiting a linear dynamic range of amplification, with R^2 values exceeding 0.99 for both regression equations. Moreover, the duplexing of both primer pairs, using Plexor technology, did not have a significant impact on the efficiency of primer pairs VertBt-F/Vert-Bt-R and PotAct-F/PotAct-R, used to quantify potato (Fig. 3). This high efficiency is crucial for meaningful quantifications of *V. dahliae* and for subsequent selections of germplasm.

In air-dried samples, *V. dahliae* is primarily in the form of microsclerotia, whereas it is in the vegetative form of conidia and mycelia in fresh samples. Four weeks following inoculations, *V. dahliae* was detected in plants of Russet Norkotah, and no evidence of infection with the pathogen was detected in plants of Ranger Russet (PED resistant) (Table 4). The lack of detection of *V. dahliae* isolate V18 in two plants of Russet Norkotah (RN4 and RN7) indicates escapes from infection and subsequent colonization by the pathogen. There was no significant difference between CFU counts and IC values, which were highly correlated (R^2 = 0.824 and P < 0.0001). Both NPX plating and Q-PCR assays successfully identified plants RN4 and RN7 as negatives, which further bolsters the conclusion that both methods are biologically correlated. It is worth mentioning that no symptoms were observed on the infected plants in the greenhouse at the time of sampling. Plants that were inoculated with *V. dahliae* isolate V18 at the same time and raised to maturity showed typical PED symptoms and died 2 to 3 weeks earlier than noninoculated control plants.

In this study, we demonstrated the effectiveness of real-time Q-PCR assays for the detection and quantification of *V. dahliae* in plant tissues. A primer pair (VertBt-F/VertBt-R) was designed that

TABLE 4. Comparison of infection coefficient (IC) and CFU values 4 weeks postinoculation of 12 Ranger Russet (potato early dying [PED]-resistant) and 12 Russet Norkotah (PED-susceptible) cultivars[a]

	Ct value[c]			
Sample[b]	*V. dahliae*	Potato	IC[d]	CFU[e]
Ranger Russet				
RR1	NA	22.7	0.0	0
RR2	NA	21.6	0.0	0
RR3	NA	22.4	0.0	0
RR4	NA	23.0	0.0	0
RR5	NA	23.5	0.0	0
RR6	NA	22.0	0.0	0
RR7	NA	22.1	0.0	0
RR8	NA	21.7	0.0	0
RR9	NA	21.5	0.0	0
RR10	NA	21.9	0.0	0
RR11	NA	22.2	0.0	0
RR12	NA	20.8	0.0	0
RRCtrl	NA	20.5	0.0	0
Russet Norkotah				
RN1	28.8	22.1	0.8	350
RN2	31.8	22.4	0.7	1,200
RN3	35.7	21.8	0.6	149
RN4	NA	21.7	0.0	0
RN5	36.8	22.4	0.6	7
RN6	29.0	21.1	0.7	704
RN7	NA	21.2	0.0	0
RN8	28.7	20.6	0.7	8
RN9	33.8	21.6	0.6	58
RN10	35.3	20.3	0.6	34
RN11	35.0	21.2	0.6	13
RN12	31.3	21.0	0.7	233
RNCtrl	NA	21.5	0.0	0

[a] Fresh stem samples weighing 50 mg were used in the real-time quantitative polymerase chain reaction (Q-PCR) assay and 50 µl of sap was used in the NPX plating assay. Inoculations were performed by dipping roots in a suspension of *Verticillium dahliae* (isolate V18) (8×10^6 conidia/ml). Spearman correlation comparing IC and CFU value: R^2 = 0.824 and P = 0.0005. NA = no amplification.

[b] Control plants (Ctrl) were dipped in water, and otherwise treated comparably to inoculated plants.

[c] Ct = cycle threshold. *V. dahliae* DNA was amplified using primer pair: VertBT-F/VertBT-R and potato (*Solanum tuberosum*) DNA was amplified using primer pair: PotAct-F/PotAct-R.

[d] IC = $Ct_{potato}/Ct_{V.dahliae}$. Limit of $Ct_{potato}/Ct_{V.dahliae}$ when the denominator tends to infinity indicated by 0.0. The number of Q-PCR amplification cycles is capped at 40 in the experiment.

[e] CFU as counted on the semiselective culture medium NPX.

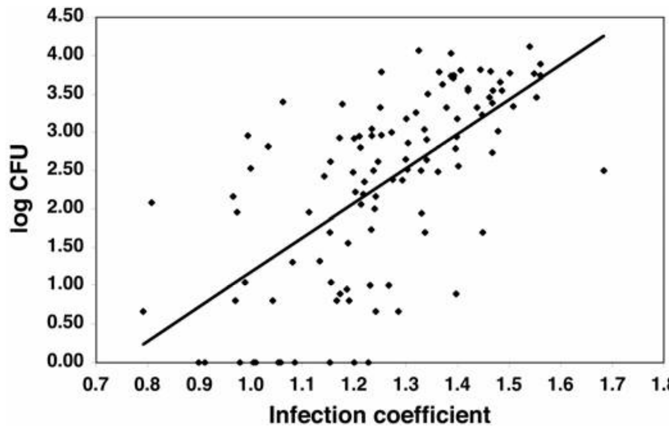

Fig. 2. Comparison of infection coefficient (IC = cycle threshold $[Ct]_{host}/Ct_{pathogen}$) and log CFU in 50-mg samples of air-dried stems of *Solanum* hybrids planted in a *Verticillium dahliae*-infested potato field and infested with *V. dahliae*-colonized rye kernels (200 CFU/g).

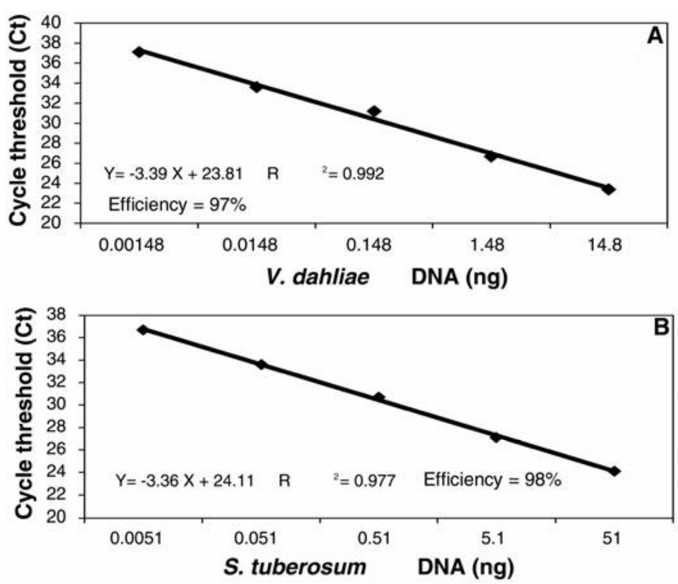

Fig. 3. Real-time quantitative polymerase chain reaction (Q-PCR) efficiency curves of a five-point serial dilution of DNA from **A,** *Verticillium dahliae* and **B,** *Solanum tuberosum* amplified in duplex using Plexor Q-PCR System (Promega Corp., Madison, WI).

selectively amplifies *V. dahliae* at DNA amounts of 148 fg while exhibiting high efficiency over six orders of magnitude. The assay also permitted the detection of *V. dahliae* of infected stems that tested negative by plating. The Q-PCR procedure reduces the time needed for the detection and quantification of *V. dahliae* from 2 weeks to 1 day and it improves the accuracy of quantification compared with plating assays. Additionally, the VertBt-F/VertBt-R primer pair provides an unambiguous detection of *V. dahliae* compared with the less discriminatory detection of multiple *Verticillium* spp. using plating assays. Further experiments currently are being conducted to identify the timing of infection, spatio-temporal distribution of the pathogen in the host, and the genetic mechanisms of susceptibility or resistance to *V. dahliae* in Ranger Russet and Russet Norkotah.

ACKNOWLEDGMENTS

This research was supported by grants from the United States Department of Agriculture–Agricultural Research Service Potato Research Program and the Wisconsin Potato and Vegetable Growers Association to Z. K. Atallah and W. R. Stevenson. We thank L. du Toit for her thorough and meticulous presubmission review of this manuscript, and the senior editor and anonymous reviewers of *Phytopathology* for helping to improve this manuscript.

LITERATURE CITED

1. Atallah, Z. K., and Stevenson, W. R. 2006. A methodology to detect and quantify five pathogens causing potato tuber decay using real-time quantitative PCR. Phytopathology 96:1037-1045.
2. Bowden, R. L., and Rouse, D. I. 1987. Effects of *Verticillium dahliae* on photosynthesis and transpiration of potato. Phytopathology 77:1703-1703.
3. Bowden, R. L., and Rouse, D. I. 1991. Effects of *Verticillium dahliae* on gas exchange of potato. Phytopathology 81:293-301.
4. Bowden, R. L., Rouse, D. I., and Sharkey, T. D. 1990. Mechanism of photosynthesis decrease by *Verticillium dahliae* in potato. Plant Physiol. 94:1048-1055.
5. Brody, H., and Carbon, J. 1989. Electrophoretic karyotype of *Aspergillus nidulans*. Proc. Natl. Acad. Sci. USA 86:6260-6263.
6. Butterfield, E. J., and Devay, J. E. 1977. Reassessment of soil assays for *Verticillium dahliae*. Phytopathology 67:1073-1078.
7. Carbone, I., and Kohn, L. M. 1999. A method for designing primer sets for speciation studies in filamentous Ascomycetes. Mycologia 91:553-556.
8. Cochran, W. G., and Cox, G. M. 1957. Experimental Designs, 2nd ed. John Wiley and Sons, New York.
9. Collins, A., Okoli, C. A. N., Morton, A., Parry, D., Edwards, S. G., and Barbara, D. J. 2003. Isolates of *Verticillium dahliae* pathogenic to crucifers are of at least three distinct molecular types. Phytopathology 93:364-376.
10. Corsini, D. L., Pavek, J. J., and Davis, J. R. 1990. Verticillium wilt resistant potato germplasm—A66107-51 and A68113-4. Am. Potato J. 67:517-525.
11. Cullen, D. W., Lees, A. K., Toth, I. K., and Duncan, J. M. 2001. Conventional PCR and real-time quantitative PCR detection of *Helminthosporium solani* in soil and on potato tubers. Eur. J. Plant Pathol. 107:387-398.
12. Cullen, D. W., Lees, A. K., Toth, I. K., and Duncan, J. M. 2002. Detection of *Colletotrichum coccodes* from soil and potato tubers by conventional and quantitative real-time PCR. Plant Pathol. 51:281-292.
13. Dan, H., Ali-Khan, S. T., and Robb, J. 2001. Use of quantitative PCR diagnostics to identify tolerance and resistance to *Verticillium dahliae* in potato. Plant Dis. 85:700-705.
14. Davis, J. R., Pavek, J. J., and Corsini, D. L. 1983. A sensitive method for quantifying *Verticillium dahliae* colonization in plant tissue and evaluating resistance among potato genotypes. Phytopathology 73:1009-1014.
15. Davis, J. R., Pavek, J. J., Corsini, D. L., Sorensen, L. H., Schneider, A. T., Everson, D. O., Westermann, D. T., and Huisman, O. C. 1994. Influence of continuous cropping of several potato clones on the epidemiology of Verticillium wilt of potato. Phytopathology 84:207-214.
16. Davis, J. R., and Sorensen, L. H. 1986. Benefits and problems associated with metam-sodium treatments on potato. Am. Potato J. 63:418-419.
17. Davis, J. R., and Sorensen, L. H. 1986. Differential effects of potato genotype and metam sodium on the population dynamics of *Verticillium* spp. Phytopathology 76:1094-1094.
18. Davis, J. R., Sorensen, L. H., Corsini, D. L., and Hafez, S. L. 1983. Effects of continuous cropping with potato genotypes on *Verticillium dahliae* and *Pratylenchus neglectus*. Phytopathology 73:958-958.
19. Davis, J. R., Sorensen, L. H., Stark, J. C., and Westermann, D. T. 1990. Fertility and management—practices to control Verticillium wilt of the Russet Burbank potato. Am. Potato J. 67:55-65.
20. Frackman, S., Ekenberg, S., Hoffmann, K., Krenke, B., Sprecher, C., and Storts, D. 2005. Plexor technology: A new chemistry for real-time PCR. Promega Notes 90:2-4.
21. Gao, X., Jackson, T. A., Lambert, K. N., Li, S., Hartman, G. L., and Niblack, T. L. 2004. Detection and quantification of *Fusarium solani* f. sp. *glycines* in soybean roots with real-time quantitative polymerase chain reaction. Plant Dis. 88:1372-1380.
22. Glass, N. L., and Donaldson, G. C. 1995. Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous Ascomycetes. Appl. Environ. Microbiol. 61:1323-1330.
23. Hall, T. A. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acid Symp. Ser. 41:95-98.
24. Hamm, P. B., Ingham, R. E., Jaeger, J. R., Swanson, W. H., and Volker, K. C. 2003. Soil fumigant effects on three genera of potential soilborne pathogenic fungi and their effect on potato yield in the Columbia Basin of Oregon. Plant Dis. 87:1449-1456.
25. Hoyos, G. P., Lauer, F. I., and Anderson, N. A. 1993. Early detection of Verticillium wilt resistance in a potato breeding program. Am. Potato J. 70:535-541.
26. Jansky, S., Rouse, D. I., and Kauth, P. J. 2004. Inheritance of resistance to *Verticillium dahliae* in diploid interspecific potato hybrids. Plant Dis. 88:1075-1078.
27. Jansky, S. H., and Rouse, D. I. 2000. Identification of potato interspecific hybrids resistant to Verticillium wilt and determination of criteria for resistance assessment. Potato Res. 43:239-251.
28. Jansky, S. H., and Rouse, D. I. 2003. Multiple disease resistance in interspecific hybrids of potato. Plant Dis. 87:266-272.
29. Jeger, M. J., Hide, G. A., VandenBoogert, P., Termorshuizen, A. J., and VanBaarlen, P. 1996. Pathology and control of soil-borne fungal pathogens of potato. Potato Res. 39:437-469.
30. Kimpinski, J., Platt, H. W., Perley, S., and Walsh, J. R. 1998. *Pratylenchus* spp. and *Verticillium* spp. in New Brunswick potato fields. Am. J. Potato Res. 75:87-91.
31. Ko, K. S., and Jung, H. S. 2002. Three nonorthologous ITS1 types are present in a polypore fungus *Trichaptum abietinum*. Mol. Phylogenet. Evol. 23:112-122.
32. Lees, A. K., Cullen, D. W., Sullivan, L., and Nicolson, M. J. 2002. Development of conventional and quantitative real-time PCR assays for the detection and identification of *Rhizoctonia solani* AG-3 in potato and soil. Plant Pathol. 51:293-302.
33. Li, K. N., Rouse, D. I., Eyestone, E. J., and German, T. L. 1999. The generation of specific DNA primers using random amplified polymorphic DNA and its application to *Verticillium dahliae*. Mycol. Res. 103:1361-1368.
34. Lynch, D. R., Chen, Q., Kawchuk, L. M., and Driedger, D. 2004. Verticillium wilt resistant germplasm-release of clone LRC18-21 and derivatives. Am. J. Potato Res. 81:295-297.
35. Mahuku, G. S., and Platt, H. W. 2002. Quantifying *Verticillium dahliae* in soils collected from potato fields using a competitive PCR assay. Am. J. Potato Res. 79:107-117.
36. Mahuku, G. S., Platt, H. W., and Maxwell, P. 1999. Comparison of polymerase chain reaction based methods with plating on media to detect and identify Verticillium wilt pathogens of potato. Can. J. Plant Pathol. 21:125-131.
37. Mercado-Blanco, J., Rodriguez-Jurado, D., Parrilla-Araujo, S., and Jimenez-Diaz, R. M. 2003. Simultaneous detection of the defoliating and nondefoliating *Verticillium dahliae* pathotypes in infected olive plants by duplex, nested polymerase chain reaction. Plant Dis. 87:1487-1494.
38. Mohan, S. K., Davis, J. R., Corsini, D. L., Sorensen, L. H., and Pavek, J. J. 1987. Comparison of methods for evaluation of potato genotypes for resistance to Verticillium wilt. Phytopathology 77:1702-1702.
39. Mohan, S. K., Davis, J. R., Corsini, D. L., Sorensen, L. H., and Pavek, J. J. 1990. Reaction of potato clones and accessions of *Solanum* spp. to *Verticillium dahliae* Kleb and its toxin. Potato Res. 33:449-458.
40. Morgan, G. D., Stevenson, W. R., MacGuidwin, A. E., Kelling, K. A., Binning, L. K., and Zhu, J. 2002. Plant pathogen population dynamics in potato fields. J. Nematol. 34:189-193.
41. Mpofu, S. I., and Hall, R. 2003. Accuracy and precision of population estimates of *Verticillium dahliae* on growth media in quantitative soil assays. Can. J. Bot. (Rev. Can. Bot.) 81:294-306.
42. Nahimana, A., Francioli, P., Blanc, D. S., Bille, J., Wakefield, A. E., and Hauser, P. M. 2000. Determination of the copy number of the nuclear rDNA and beta-tubulin genes of *Pneumocystis carinii* f. sp *hominis* using PCR multicompetitors. J. Eukaryot. Microbiol. 47:368-372.

43. Nicot, P. C., and Rouse, D. I. 1987. Relationship between soil inoculum density of *Verticillium dahliae* and systemic colonization of potato stems in commercial fields over time. Phytopathology 77:1346-1355.
44. Paris, R., and Lamattina, L. 2002. Increased ratio of mitochondrial rDNA to cytoplasmic rDNA during zoosporic and germinating cyst stages of the life cycle of *Phytophthora infestans* (Mont.) de Bary. Can. J. Microbiol. 48:268-274.
45. Pegg, G. F., and Street, P. F. S. 1984. Measurement of *Verticillium albo-atrum* in high and low resistance hop cultivars. Trans. Br. Mycol. Soc. 82:99-106.
46. Powelson, M. L., and Rowe, R. C. 1993. Biology and management of early dying of potatoes. Annu. Rev. Phytopathol. 31:111-126.
47. Ristaino, J. B., Madritch, M., Trout, C. L., and Parra, G. 1998. PCR amplification of ribosomal DNA for species identification in the plant pathogen genus *Phytophthora*. Appl. Environ. Microbiol. 64:948-954.
48. Rowe, R. C., Davis, J. R., Powelson, M. L., and Rouse, D. I. 1987. Potato early dying—causal agents and management strategies. Plant Dis. 71:482-489.
49. Rowe, R. C., and Powelson, M. L. 2002. Potato early dying: management challenges in a changing production environment. Plant Dis. 86:1184-1193.
50. Rozen, S., and Skaletsky, H. 2000. Primer3 on the WWW for general users and for biologist programmers. Pages 365-386 in: Bioinformatics Methods and Protocols: Methods in Molecular Biology. S. Krawetz and S. Misener, eds. Humana Press, Totowa, NJ.
51. Sadras, V. O., Quiroz, F., Echarte, L., Escande, A., and Pereyra, V. R. 2000. Effect of *Verticillium dahliae* on photosynthesis, leaf expansion and senescence of field-grown sunflower. Ann. Bot. 86:1007-1015.
52. Saeed, I. A. M., MacGuidwin, A. E., Rouse, D. I., and Sharkey, T. D. 1999. Limitation to photosynthesis in *Pratylenchus penetrans*- and *Verticillium dahliae*-infected potato. Crop Sci. 39:1340-1346.
53. Schweigkofler, W., O'Donnell, K., and Garbelotto, M. 2004. Detection and quantification of airborne conidia of *Fusarium circinatum*, the causal agent of pine pitch canker, from two California sites by using a real-time PCR approach combined with a simple spore trapping method. Appl. Environ. Microbiol. 70:3512-3520.
54. Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F., and Higgins, D. G. 1997. The CLUSTAL_X windows interface: Flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. 25:4876-4882.
55. Thompson, J. D., Higgins, D. G., and Gibson, T. J. 1994. Clustal-W—Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22:4673-4680.
56. Tooley, P. W., Bunyard, B. A., Carras, M. M., and Hatziloukas, E. 1997. Development of PCR primers from internal transcribed spacer region 2 for detection of *Phytophthora* species infecting potatoes. Appl. Environ. Microbiol. 63:1467-1475.
57. Tooley, P. W., Carras, M. M., and Lambert, D. H. 1998. Application of a PCR-based test for detection of potato late blight and pink pot in tubers. Am. J. Potato Res. 75:187-194.
58. Trout, C. L., Ristaino, J. B., Madritch, M., and Wangsomboondee, T. 1997. Rapid detection of *Phytophthora infestans* in late blight-infected potato and tomato using PCR. Plant Dis. 81:1042-1048.
59. Valsesia, G., Gobbin, D., Patocchi, A., Vecchione, A., Pertot, I., and Gessler, C. 2005. Development of a high-throughput method for quantification of *Plasmopara viticola* DNA in grapevine leaves by means of quantitative real-time polymerase chain reaction. Phytopathology 95:672-678.
60. Wen, K., Seguin, P., St-Arnaud, M., and Jabaji-Hare, S. 2005. Real-time quantitative RT-PCR of defense-associated gene transcripts of *Rhizoctonia solani*-infected bean seedlings in response to inoculation with a non-pathogenic binucleate *Rhizoctonia* isolate. Phytopathology 95:345-353.
61. Wheeler, T. A., Madden, L. V., Rowe, R. C., and Riedel, R. M. 2000. Effects of quadrat size and time of year for sampling of *Verticillium dahliae* and lesion nematodes in potato fields. Plant Dis. 84:961-966.
62. Wheeler, T. A., Rowe, R. C., Riedel, R. M., and Madden, L. V. 1994. Influence of cultivar resistance to *Verticillium* spp. on potato early dying. Am. Potato J. 71:39-57.
63. Winton, L. M., Manter, D. K., Stone, J. K., and Hansen, E. A. 2003. Comparison of biochemical, molecular, and visual methods to quantify *Phaeocryptopus gaeumannii* in Douglas-Fir foliage. Phytopathology 93:121-126.
64. Winton, L. M., Stone, J. K., Watrud, L. S., and Hansen, E. M. 2002. Simultaneous one-tube quantification of host and pathogen DNA with real-time polymerase chain reaction. Phytopathology 92:112-116.
65. Zuker, M., Mathews, D. H., and Turner, D. H. 1999. Algorithms and Thermodynamics for RNA Secondary Structure Prediction: A Practical Guide in RNA Biochemistry and biotechnology. J. Barciszewski and B. F. C. Clark, eds. Kluwer Academic Publishers, Dordrecht, the Netherlands.